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Antimicrobial Effectiveness of Ethanol Extract of Annona Muricata L (Soursop Leaves) Against Escherichia Coli Bacteria In Vitro

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ABSTRACT

Introduction: Infectious diseases are health problems that develop over time. Various microorganisms, such as bacteria, viruses, fungi, and protozoa, cause infections. One of the bacteria that caused the infection was Escherichia coli. Gram-negative bacteria can cause infections in the digestive tract, namely diarrhoea. One of the therapies for diarrhoea is antibiotics. However, consuming antibiotics in the long term can increase the risk of antibiotic resistance, urticaria, headaches and nausea. One of the traditional medicines used to relieve diarrhoea symptoms is soursop leaves (Annona muricata L), which have various secondary metabolite compounds that can kill bacteria.
Objectives and Methods: This study aims to determine the antibacterial activity of soursop leaves (Annona muricata L) against Escherichia coli. The ultrasonic method is used to extract soursop leaves. Test antibacterial activity using the healthy diffusion method and determine the minimum inhibitory concentration using the diffusion method. Results: This study shows that soursop leaves have antibacterial activity through healthy diffusion tests with an average inhibition zone at a concentration of 0.5% of 6.59 mm, a concentration of 1% of 12.3 mm.
Results: the inhibition zone given ethanol extract of soursop leaves (EtAML) 0.5% has a moderate inhibition zone result, and EtAML 1% has a strong inhibition zone result.
Conclusion: This study concludes that soursop leaves (Annona muricata L) have antibacterial activity against Escherichia coli with the results of ethanol extract of soursop leaves (EtAML) 0.5% having a moderate inhibition zone result and EtAML 1% having a solid inhibition zone result.

Introduction

The prevalence of diarrhoea cases worldwide increases every year. According to WHO data, the prevalence of diarrhoea disease worldwide is 1.7 billion and ranks 2nd as the cause of death for children under five years of age. (WHO, 2023). According to Riskesdas Data, in 2018, there was an increase in the prevalence of diarrhoea cases by 6.8% in Indonesia (Riskesdas, 2018). *Diarrhoea* is a disease characterized by the consistency of defecation in liquid form three or more times compared to the average amount (Selomo & La Ane, 2019). Diarrhoea is a disease with relatively high mortality and morbidity cases. In the world, 1.7 billion cases of diarrhoea occur each year.

In contrast, according to Riskesdas, in 2018, the prevalence of diarrhoea in Indonesia was recorded as the highest in children aged 5-14 years, namely 182,338 (6.2%) (Ministry of Health, 2019). Most cases of diarrhoea 90% are caused by infectious agents, including pathogenic bacteria that are contaminated through contaminated food or water, such as gram-negative pathogenic bacteria such as Salmonella sp, Escherichia coli, Pseudomonas aeruginosa Sp, Shigella

dysenteriae Sp (Rini & Rohmah, 2020). One of the therapies for diarrhoea is by administering antibiotics. Antibiotics are chemical compounds derived from organisms such as bacteria and fungi that aim to inhibit the growth of other microorganisms, especially pathogenic bacteria. However, consuming antibiotics in the long term can increase the risk of antibiotic resistance, which results in the ineffectiveness of previously circulating antibiotics (Sudigdoadi, 2015). In addition to resistance, giving antibiotics can also have side effects, such as previous studies showing that giving antibiotic therapy can cause side effects such as urticaria (13.72%), headaches (1.96%) and nausea (1.96%) (Ratman, 2019). Antibiotics that are not consumed according to recommendations can increase cases of antibiotic resistance. According to data reported by Cancer for Disease Prevention, every year, 13,000 patients die due to resistance to pathogenic bacteria. This data is also supported by the small discovery of antibiotics inversely proportional to cases of resistance to pathogenic bacteria. Cases of antibiotic resistance include those caused by bacterial resistance, such as Staphylococcus aureus (S. aureus) (Setiawati, 2015) and Escherichia colic (Syafriana, 2020). Antimicrobials are compounds that play a role in suppressing or eradicating the growth of pathogenic bacteria that develop and cause infections when they enter the host tissue (Jawetz et al., 2019). *Antimicrobials* are compounds used to control the growth of harmful microbes. Controlling the growth of microorganisms aims to prevent the spread of infectious diseases by eradicating microorganisms in infected hosts and preventing decay and destruction of materials by microorganisms (Habibi et al., 2022). In addition to antibiotics, plant metabolite compounds can inhibit bacterial growth. Metabolite compounds that inhibit or kill bacterial growth, known as antimicrobials, include saponins, tannins, flavonoids, xanthones, terpenoids, and alkaloids (Amalia et al., 2018).

Indonesia has a myriad of biodiversity that has been used as medicinal plants for generations. Medicinal plants have long been used as traditional medicine in parts of the world, especially the third world, which has limited access to public health services. The trend of consuming medicinal plants is increasing, so further research is needed to provide information in the form of compounds contained in medicinal plants that are efficacious for the body or toxic so that their use is minimized (Apriliana & Syafira, 2016).

Among this biodiversity, the community often uses soursop leaves (Annona muricata L) as medicinal plants. Soursop (Annona muricata L) thrives in several countries in the Americas, Africa and Asia, including Indonesia. The use of soursop in each region varies, such as Haitian society using it as an anti-diarrhoea, fever, flu, lactation for breastfeeding mothers, heart, anti-parasite, wounds, and seizures. In Mexico, it is a fever reducer; in Brazil, it is an anti-ulcer, cardiovascular therapy, bronchitis, diabetes, dysentery, anti-diarrhea, preventing bleeding, and fever. Ecuador as an analgesic drug, and several countries in the African continent as a fever medicine (Hasmila, 2015). However, in previous literature studies, there has been no research showing the effectiveness of soursop leaves as an antibacterial against pathogenic bacteria Escherichia coli.

Material and Methods

1. Tools and Materials

The tools used in this research are incubator, biological safety cabinet (BSC), hot plate (thermo scientific-care), magnetic stirrer, vortex, stirring rod, Erlenmeyer (Pyrex), test tube (Pyrex), rack, glass measuring (Pyrex), dropper pipette, arlogy glass, analytical balance (Acis AD-600i), petri dish, beaker glass (Pyrex), horn spoon, gloves, mask, spatula, tissue, lighter, autoclave (GEA YX-280D), tube needle, spirit lamp, funnel (Pyrex), aluminium foil, plastic wrap, ultrasonic cleaner, scissors, rotary evaporator, caliper, refrigerator, sieve, micropipette, blender, and porcelain cup.

The materials used in this research were the soursop Leaves (*Annona muricata L.*) as research samples, sterile distilled water, 70% ethanol, the drug chloramphenicol, Escherichia coli

bacteria ATTC 25922, Nutrient Agar (NA), Nutrient Broth (NB), DMSO 10%, label paper, cotton, BaCl2, H2SO4, and NaCl.

2. Methods

a. Extraction of soursop Leaves *(Annona muricata L.)*

The extract materials used in this study were 1 kg of soursop leaves and 1 kg sorted from their branches. The soursop leaves were then air-dried on the edge of the terrace to avoid exposure to direct sunlight. The air-drying process lasted a week until the leaves were dehydrated. The dried soursop leaves were then blended into powdered simplicia. The simplicia was then sieved with a sieve no. 4 Mesh. The maceration method extraction process was done by soaking 200 grams of soursop leaf powder with 600 mL of 96% ethanol, then tightly closed and left for 3 days, avoiding direct sunlight. During the soaking process, it was stirred occasionally to increase the diffusion of the soursop leaf simplicia into the ethanol solution.

The simplicia mixed with 96% ethanol for three days was then filtered using a Buchner funnel and squeezed to obtain the first macerate. The remaining dregs were soaked again with 96% ethanol for three days until the second macerate was obtained. The first macerate was mixed with the second macerate. Then, the concentration process was carried out using a rotary evaporator at a temperature of 65C to obtain a thick soursop leaf extract. The extract that had been obtained was then tested for phytochemicals to determine the content of phenolic compounds in the extract.

b. Testing of phytochemical compounds in soursop leaf extract (*Annona muricata L.*)

Phytochemical compound tests are carried out by aligning the test tubes in a rack; each tube is added 0.5 grams of soursop leaf extract, and reagents are added according to each test. Alkaloid test: 0.5 mL of 1% HCL is added, and two drops of Dragendorf reagent are positive if orange appears. Flavonoid test: Add sufficient Mg powder, 2 mL of hot water, four drops of 37% HCL, and four drops of 96% ethanol, then shake. Positive Flavonoid if yellow, red, or orange appears. Tannin test: Add two drops of 1% FeCl3 and 2 mL of distilled water, positive if bluish green. Saponin test: Add 0.5 mL of hot water and shake for 1 minute, and if there is foam, add 1 N HCL and positive if it contains saponin if the foam formed lasts for 10 minutes with an average height of 1-3 cm. Steroid and Triterpenoid Test: Add 0.5 mL CHCL3, 0.5 mL anhydrous acetic acid, and 2 mL H2SO4 4N. Positive for triterpenoids if a reddish-purple colour appears and steroids if a green or blue colour appears.

c. Sterilization and Media Preparation

The media used in the study were 5.6 grams of nutrient agar plate (NA) media dissolved in 250 mL of distilled water on a hotplate magnetic stirrer. The media that had been dissolved in the Erlenmeyer flask was then wrapped in brown cardboard with a petri dish, tube, and one needle and put into an autoclave at 121°C at a pressure of 1 atm. The sterilized media was then poured into a petri dish until solid.

d. Bacterial culture and identification

The NA media was inoculated with bacterial culture samples and then incubated at 37°C for 24 hours. The bacteria that grew on the NA media were taken from their colonies to undergo gram staining and biochemical identification tests. Gram staining was done by taking a few bacteria from the colony using an incandescent ose and slowly spreading them on the object glass. After that, fixation was carried out by passing it through the fire two to three times. Next, the slide is dripped with crystal violet and waited for 1 minute, then rinsed with running water, dripped with iodine for 1 minute, dripped with alcohol for 30 seconds and dripped with safranin for 1 minute. After being coloured, the dried slide is observed under a microscope with a magnification of 100x. The gram-negative bacteria group will be red, while the gram-positive bacteria group will be purple.

e. Making Bacterial Media Wells

Nutrient agar plate (NA) media is inoculated using a cotton swab and left for 5 minutes. After 5 minutes, 4 wells are made using a cork punch, each of which is pipetted with 100 µL. The first hole contains 96% ethanol (K-), the second hole is 0.5% antibiotic suspension (K+), the third hole is 0.5% soursop leaf extract suspension (P1), and the fourth hole is 1% soursop leaf extract suspension (P2). After all were pipetted, the NA media was incubated for 24 hours at 37C.

f. Data Analysis Method

The study's results were an average of measuring the diameter of the antibacterial inhibition zone of soursop leaves using a ruler or calliper. It is said that the inhibition zone is a zone or area that is not overgrown with bacteria, which is marked by a clear zone surface around the sound hole that has been suspended with ethanol, antibiotics, and soursop leaf ethanol extract (EtAML) compared to the surface where bacteria grow. The results of the measurement of the diameter of the inhibition zone can be calculated using the formula: Dz = (Dv-Dc) + (Dh-Dc) 2 Dv: Vertical diameter of the inhibition zone that is not overgrown with bacteria Dh: Horizontal diameter of the inhibition zone that is not overgrown with bacteria Dc: Diameter of the disc or well hole The results of the measurement of the diameter of the area of the inhibition zone between the treatments K- (Ethanol), K+ (Antibiotic), P1 (EtAML 0.5%) and P2 (EtAML 1%) are calculated as the average value which can then be concluded based on the category of inhibitory strength and the difference in the average diameter of the inhibition zone of each treatment group.

Results and Discussion

The sensitivity test is a test to measure the level of sensitivity of a bacteria to antibacterial substances. The bacterial sensitivity test method aims to determine natural products that have the potential as antimicrobial materials and can inhibit growth or kill bacteria based on different concentrations, starting from low concentrations. (Siti, 2013). Antibacterial activity tests can be carried out using the diffusion method (disc diffusion test) and dilution (dilution test). The diffusion method is carried out by measuring the diameter of the clear zone to indicate the presence of a response to bacterial growth inhibition by an antibacterial compound in the extract. The principle of the diffusion method is the diffusion of compounds contained in antibacterials in solid media or plates that have been previously inoculated with isolates or sources of bacteria. There are three diffusion methods: the cylinder, the hole or well, and the disc paper method. The disc paper method is carried out by placing disc paper or plates containing antibiotics on the surface of the agar media that has been inoculated with bacterial isolates. After 18-24 hours of incubation, the precise area or zone around the disc or well is observed to observe the growth activity of the antibacterial inhibition zone.

Meanwhile, the hole or healthy method involves making a hole in the agar media that has been inoculated with bacteria, which is then injected with extracts with different concentrations (Nurhayati et al., 2020) after inoculation at a temperature and time that matches the test microbe. Observation involves seeing whether or not there is an inhibition zone with a specific diameter (Prayoga, 2013).

The antibacterial activity method in the study was the healthy method. This method was chosen because the sound method has the advantage of being easier to measure the area of the inhibition zone formed. In addition, the sound method also increases bacterial activity, concentrating on the upper surface of the nutrient agar and penetrating the bottom of the media. The sound method is also more practical and straightforward, especially the replication of each treatment in large quantities (Darmawati, 2022). The sound method is done by making a well in the media inoculated with bacteria. The holes or wells were then given four different treatments, namely ethanol (A), 0.5% standard antibiotic (B), 0.5% soursop leaf ethanol extract (EtAML) (C) and 1% EtAML (D). The treatments injected into the wells were then diffused into the nutrient agar (NA) media inoculated with bacteria. Antibacterial activity can be indicated by the presence of an inhibition zone seen by forming a clear area around the well (Mengko et al., 2022). Previous

literature studies stated that antibacterial activity is categorized as weak if the diameter of the inhibition zone formed is less than 5 mm, categorized as moderate activity if the diameter of the inhibition zone formed is 5-10 mm, vigorous activity if the diameter formed is 10-19 mm, and extreme activity if the diameter formed is above 20 mm (Geofani et al., 2022). The observations in Table 1 showed that the average area of the ethanol inhibition zone (A) had a smaller inhibition zone diameter compared to other treatments. This is because ethanol cannot diffuse into the media. After all, ethanol is a local antiseptic (Abd El-Gawad et al., 2014).

Tabel 1. Phytochemical Screening Results of Sambung Nyawa Leaf Extract

Treatment	Means Replication 10x	Result
Et (A)	1,35	Weak
Antibiotic Amoxilin 0,5% (B)	18,37	Strong
EtAML 0,5% (C)	6,59	Medium
EtAML 1% (D)	12,3	Strong

The study results in Table 1 show that control group B, or wells suspended with 0.5% amoxicillin antibiotic, have a more expansive inhibition zone than other treatments. Amoxicillin antibiotic is included in the strong category in inhibiting bacteria with an average diameter of 18.37 mm. This is because amoxicillin antibiotics can inhibit the growth of gram-positive and gram-negative bacteria, causing a more expansive inhibition zone (Heningtyas & Hendriani, 2018). Amoxicillin antibiotic is included in the penicillin group, whereas penicillin is included in the β-lactamase group, which targets damage to the cell membrane (Anggita et al., 2022). B-lactamase works by disrupting the formation of bacterial membrane synthesis by inhibiting the formation of peptidoglycan synthesis in forming the bacterial membrane layer. This step can damage the bacterial membrane so that it can interfere with bacterial growth (Zuhriyah et al., 2018). The study results in Table 1 show that the treatment of 1% soursop leaf ethanol extract (EtAML) or group D has a wider inhibition zone diameter of 12.3 mm compared to 0.5% EtAML or group C. The higher the concentration, the larger the inhibition zone formed. Bacterial growth will mainly decrease along with the increasing concentration of antibacterial agents. The higher the concentration of the extract, the higher the ability of the compounds contained in the extract to inhibit bacterial growth. This high ability is due to the more excellent antibacterial compounds contained in the extract, making it easier for the compounds to penetrate the bacteria (Lingga et al., 2016), which can ultimately damage the walls of the bacteria (Anggita et al., 2022).

Soursop leaves are plants that have long been used in medicine and have been shown to have antibacterial activity. This is because soursop leaves contain tannins, flavonoids, polyphenols, saponins and essential oils (Najib et al., 2022). The study results showed that soursop leaf extract contained positive compounds of alkaloids, saponins, flavonoids, steroids, and tannins. The results of this study are from previous literature studies (Fikri et al., 2019). The mechanism of inhibition of bacterial growth by antibacterial compounds can damage the walls or membranes of bacteria, change the permeability of the cytoplasmic membrane, cause the release of food from the cell, change protein and nucleic acid molecules, inhibition of enzyme activity and inhibition of enzyme activity and nucleic acids and proteins (Anggraini et al., 2019). The study results in Table 1 show that EtAML 1% (D) is more effective than the negative control (ethanol). This is because of the membrane layer of gram-negative bacteria. However, the membrane layer is thick because it is composed of many lipids. However, it also contains a lot of porin protein, which acts as a transport of active compounds into bacteria. The presence of this protein causes active compounds such as tannins, saponins, and flavonoids to quickly enter cells, thereby damaging the activity of enzyme formation, which is helpful for bacterial growth and development. (Nur, 2017). High lipid content also increases cell damage because membrane lipids can increase the permeability of active compounds such as tannins, saponins and flavonoids into cells. (Anggita et al., 2022). Tannin compounds contained in soursop leaf ethanol extract can

interfere with forming proteins or enzymes in bacteria. Saponin and flavonoid content can interfere with the diffusion process by breaking down membrane lipids, thereby disrupting the food ingredients needed for the growth and reproduction of bacterial cells (Masloman, 2016).

Conclusion

The results showed that an ethanol extract of soursop leaves at a concentration of 1% had a better ability to inhibit bacterial growth than a concentration of 0.5%. However, its effectiveness still needed to be comparable to the standard antibiotics used in this study. This finding indicates that although soursop leaf extract can be a promising alternative as an antimicrobial agent, further research is still needed to improve its effectiveness in competing with established antibiotic drugs.

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